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MYC TRANSCRIPTION FACTORS: KEY REGULATORS BEHIND ESTABLISHMENT AND MAINTENANCE OF PLURIPOTENCY

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Summary

The interplay between transcription factors, epigenetic modifiers, chromatin remodelers and miRNAs form the foundation of a complex regulatory network required for establishment and maintenance of the pluripotent state. Recent work indicates that Myc transcription factors are essential elements of this regulatory system. Despite numerous reports however, aspects of how Myc controls self-renewal and pluripotency remain obscure. Here, we review evidence supporting the placement of Myc as a central regulator of the pluripotent state and discuss possible mechanisms of action.

Keywords

Myc; pluripotent; self-renewal; reprogramming; embryonic stem cells; induced pluripotent stem cells

Our understanding of pluripotent cells derived from the inner cell mass of blastocyst-stage embryos has been guided by the characterization of embryonic stem cells (ESCs). Under the appropriate culture conditions, ESCs can be maintained as a stable self-renewing population with developmental potential comparable to that of pluripotent cells in the inner cell mass (ICM) [1–3]. Recently, ectopic expression of transcription factors including Oct4, Sox2, Klf4 and c-myc were shown to reprogram somatic cells to a pluripotent state that closely resemble ESCs. These cells are referred to as induced pluripotent stem cells (iPSCs) [4, 5]. Few differences are evident between ESCs and iPSCs as judged by global gene expression and epigenetic signatures [6, 7]. Both pluripotent stem cell (PSC) populations can form embryonic germ layer derivatives *in vitro* and *in vivo* and possess long-term self-renewing capacity in culture [4, 8–10]. These characteristics make PSCs an excellent model for early embryonic development and a promising source of material for the development of cell therapies [11, 12]. Since iPSCs can be readily derived from somatic cells, it may be feasible to generate patient-specific stem cells that can then be used as part of a cell therapy strategy to cure disease and repair injury. Transplantation of a patient's cells would offer many advantages. For example, they would avoid immunological complications associated with the transplantation of foreign donor cells. Since iPSCs are derived from somatic cells and do not involve embryonic material, they bypass the ethical concerns associated with use of ESCs. Before the promise inherent to both ESCs and iPSCs can be fully realized, it is

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critical to fully understand the mechanisms that underpin their establishment and maintenance. This review will focus on what we currently know about the role of Myc in maintenance of pluripotent cells and its functions in cellular reprogramming.

Myc: An Introduction

The three major Myc family members, c-myc, N-myc and L-myc have well-established roles in cell growth, proliferation, cytoskeletal structure, cellular adhesion and motility, differentiation, and apoptosis [13–17]. Myc factors also have well-established transforming capacity of primary cells *in vitro* and they frequently act as oncogenes in tumor development [17, 18]. The Myc family members share significant protein sequence similarity and are characterized as belonging to the basic helix-loop-helix family of transcription factors. In different contexts, Myc can activate or repress target genes by binding to regulatory regions in a sequence-dependent or independent manner [17, 19–21]. Trans-activation by Myc often occurs through canonical and non-canonical E-box sequences (CACGTG/CANNTG) [22, 23]. However, their trans-activation potential is fairly weak, in general typically resulting in only small changes in target gene expression [22, 24, 25].

Myc family-members heterodimerize at E-boxes with their binding partner, Max [26–29]. Recent evidence also suggests that some E-box-independent activities of Myc can be associated with transcriptional repression, regulation of apoptosis, control of epigenetic modifications, binding to pre-replicative complexes at replication origins, post-transcriptionally controlling protein expression and activation of RNA polymerase I and III-dependent transcription. In these contexts, Myc functions independently of Max [30–37]. Little is known regarding mechanisms of Myc-mediated repression. Perhaps the best-characterized example involves E-box independent repression of Miz-1 and its targets [31].

In addition to the functions mentioned above, Myc potentially regulates large numbers of genes. Global studies indicate that c-myc binds both genic and intergenic regions to control the expression of more than 10% of all genes as well as non-coding (nc) RNAs [19, 22, 38].

Myc: Critical for Embryonic Development?

Deciphering roles for Myc transcription factors in the peri-implantation embryonic stage from which ESCs are derived has been challenging because they display functional redundancy and overlapping expression patterns during that timeframe [39, 40]. This is likely to explain the absence of distinct developmental defects during pre- and peri-implantation stages of development following knockout of Nor c-myc individually. N-myc null embryos die at approximately E10.5–12.5, displaying defects in the developing heart, lung, gut and neural system [41, 42]. c-myc knockout embryos arrest by E10.5, displaying size reduction in addition to malformations in the developing heart, neural tube, vascular and hematopoietic systems, as well as defects in the turning of the embryo [43, 44]. Trumpp and coworkers suggested that the lethality due to c-myc inactivation was primarily due to placental and hematopoietic deficiencies, as residual N-myc activity could possibly substitute in other contexts [45].

More recently, we and others have demonstrated that the presence of either c- or N-myc is essential for early embryonic development [46, 47] and that both are required for ESC (46,47) and iPSC (46) self-renewal. Myc seems to promote pluripotency at multiple levels. The first molecular targets shown to be critical for Myc's role in self-renewal were recently identified and involve the repression of differentiation genes such as GATA6 and the activation of miRNAs that control the cell cycle (46). Together, these studies underscore the importance of c-myc or N-myc in embryonic development at the time of implantation. As L-myc failed to compensate for c- and N-myc, these studies also reemphasize the differential

roles held by c-myc and N-myc versus L-myc in embryonic development [46]. It was previously shown that although L-myc is expressed in multiple organs in the developing embryo, c-myc and/or N-myc may functionally substitute as L-myc null animals are viable with no apparent abnormalities at birth [48]. L-myc is also the least tumorigenic of the three paralogs, and opposes the effect of c-myc with some target genes [29, 49, 50]. Max deletion is lethal at approximately E5.5, and questions remain about whether N- and c-myc are functionally redundant in all contexts, particularly with respect to specification of embryonic germ layer derivatives [51].

Regulation of PSCs: Complex Circuitry

Self-renewal and pluripotency in PSCs are governed by the actions of a complex regulatory circuit. Signaling molecules such as LIF and BMPs stimulate downstream transcription factor activity to generate regulatory loops comprised of transcription factors and their targets – these include additional transcription factors, signaling components and miRNAs [52–57]. Global studies suggest that constant expression of the fundamental components of this regulatory network is strictly controlled by perpetuation of feed-forward loops. This generates a circuit that may be further fine-tuned by the action of miRNAs [55–58]. Protein-protein interactions that mediate complex formation facilitate the recruitment of additional regulators to target gene promoters to further expand the circuit [59, 60]. It is generally thought that maintenance of this network serves to inhibit differentiation while promoting self-renewal [55, 60–63].

Oct4, Sox2 and Nanog were the first components of the core transcriptional regulatory circuitry identified using genome-wide location analyses in human and mouse ESCs [55, 63]. These transcription factors are thought to regulate hundreds of target genes in pluripotent cells, indicative of their pivotal role in pluripotency. Subsequent reports provide evidence for a larger network consisting of additional transcription factors including Nac1, Dax1, and Zfp281 [58]. Together, these key transcriptional regulators are thought to maintain expression of protein products and miRNAs required for maintenance of pluripotency, while simultaneously imposing a blockade on those that would result in lineage specification. Although this suggests dual potential of the transcriptional circuitry to activate and repress target genes to potentiate the pluripotent state, direct evidence for functional activation or repression of those hundreds of target genes by each central regulator is mostly lacking [55, 58]. As a transcription factor with mRNA and ncRNA targets and as a modulator of the epigenome required by many stem cell populations [19, 22, 34, 64], Myc holds unique potential as a key controller of the regulatory processes mentioned above. Significant effort has therefore been extended into examination of Myc's role in the PSC circuit.

Myc: Pleiotropic regulator of the PSC state

Substantial progress has been made to define Myc's importance in various adult stem cell and multipotent progenitor populations. In the absence of N-myc, neural progenitors are not maintained, as proliferation is inhibited while differentiation ensues [65]. N-myc also plays a similar role in the developing lung where it regulates proliferation to maintain progenitor cells [66]. The survival of dormant hematopoietic stem cells (HSCs) appears to be Myc-independent. However, loss of both c- and N-myc affects the survival, proliferation and differentiation of cycling HSCs [67]. In this case, deletion of both family members was required to uncover roles for myc. While loss of N-myc leads to no hematopoietic defect, c-myc deficient HSCs differentiate inappropriately due to modified interactions with the niche [68]. As c-myc seems to perform a dominant role, this implies some functional hierarchy

between both family members. In another example, c-myc regulates interactions between epidermal stem cells and the niche [69].

Several lines of evidence support Myc's involvement in establishment and maintenance of PSC populations (Figure 1). Initially, c-myc was implicated in maintenance of mESC self-renewal where it is a target of LIF/Stat3 signaling [64]. Following LIF withdrawal, Myc levels decline but if Myc activity is maintained by expression of a stable mutant, ESCs become LIF-independent. Moreover, expression of a dominant-negative mutant results in loss of self-renewing capacity [64]. On the other hand, although c- and N-myc single knockout mESCs are able to self-renew [42, 43, 70], c-myc$^{-/-}$ cells proliferate more slowly and have defects in vascular and hematopoietic differentiation *in vitro*. This observation was recapitulated *in vivo* using teratoma assays [70]. As expected, inactivation of both c- and N-myc produced a more deleterious effect. Simultaneous deletion of both family members resulted in loss of self-renewal and ensuing differentiation [46, 47]. Recent studies have found that Myc inhibits differentiation to primitive endoderm through repression of GATA6 [46]. Specifically, upon co-deletion of c- and N-myc in iPSCs and ESCs cultured in LIF, cells primarily differentiate into primitive endoderm at the expense of other lineages [46]. Strikingly, multi-lineage differentiation capacity is lost even when cells are cultured in the absence of LIF. In support of this, activation of Myc blocks the primitive endoderm specification *in vitro* as well as that on the surface of embryoid bodies [46]. Similar to the aforementioned observations in embryonic development, L-myc is unable to substitute for the other family members to maintain self-renewal and pluripotency [46]. This is in opposition to the capacity of L-myc to increase reprogramming efficiency in a manner similar to c-myc [71].

Contribution to the PSC Regulatory Circuitry

Several studies have identified genome-wide Myc targets in PSCs [46, 58, 73–75]. Although these target genes are known to be involved in regulation of the cell cycle and metabolism, this information offered no mechanistic insight into how Myc maintains or establishes the pluripotent state. Most notably absent from initial global studies were genes with well-established roles in pluripotency or lineage specification.

The mechanism for how Oct4, Sox2, Klf4 and Nanog regulate pluripotency is based, in part, on the identification of genes they bind *in vivo*. For example, binding to active genes indicates a role in positive regulation while binding to silent genes suggests repression. By the same type of approach, Oct4, Sox2, Klf4 and Nanog also seem to auto-regulate their own expression. Similarly, Myc is implicated in cell cycle control, metabolic regulation and inhibition of differentiation based on target gene identification [46, 55, 58, 63]. However, instead of being implicated in the Oct4, Sox2, Nanog, and Klf4 circuit, Myc is instead connected to a distinct regulatory network with Trim28 and Cnot3 [58, 73, 75, 76].

Several studies have shown that Myc binds to the regulatory regions of thousands of genes, implying possible roles as a more global regulator of transcription [22, 22, 38, 77]. More than 1600 direct c-myc target genes (www.myccancergene.org) and miRNAs have been identified in somatic cells and cancer cell lines. These targets have roles in cell cycle regulation, metabolism, chromatin maintenance, RNA and protein synthesis and processing, cellular structure and adhesion. Even this is still likely to be an under-estimation of true numbers of c-myc targets as Amati and colleagues have shown that it may regulate 10–15% of all genes [22]. This has held true in PSCs where global studies have collectively identified upwards of 3000 targets [58, 73–75]. Interestingly, Myc is also implicated to regulate PolII pause-release at ~30% of actively transcribed genes in PSCs through the recruitment of P-TEFb [78]. In addition to local targets, recent studies have also indicated

that Myc may also bind to and regulate expansive chromatin domains to functionally maintain euchromatin [19, 79]. As ESCs are unique in their state of expansive euchromatic regions, this potential held by Myc could establish its role as a global controller of the epigenetic state of PSCs (discussed later).

Regulation of the PSC Cell Cycle

The unlimited capacity and rate of PSC proliferation is underscored by their unique cell cycle structure and regulation. To divide with generation times of approximately 8–12h, murine PSCs maintain a cell cycle profile with the majority of the population (~60%) in S phase interspersed by short gap phases [80]. Myc impacts the cell cycle structure in a multitude of ways and through a number of cell cycle regulators, including cyclins D2, E and A [58, 73, 75]. Elevated Myc activity accounts for the increased activity of these regulators and consequently, the cell cycle structure and rapid cell division rates in PSCs [81]. Furthermore, expression of the CDK inhibitors, $p21^{Cip1}$, $p27^{Kip1}$ and INK4 family members in PSCs is suppressed. Myc is also likely to have a role in regulation of these genes as it can interfere with their activation [21, 80, 82].

Murine PSCs also appear to cycle independently of mitogenic signaling pathways, much in the same way as tumor cells. Mechanistically, this seems to be related to the constitutive hyper-phosphorylation of the retinoblastoma (Rb) tumor suppressor family [80, 83, 83]. Rb is the regulator of a cell cycle gateway in G1, known as the restriction point, which normally connects mitogenic signaling pathways to the cell cycle machinery. Inactivation of Rb by phosphorylation bypasses the restriction point [84], enabling PSCs to cycle in an unrestrained manner. Also consistent with inactive Rb is the constitutive activation of E2F transcription factors. This facilitates expression of E2F targets, many of which include cellular proliferation genes [80, 81]. Both c- and N-myc physically interact with Rb [85], and in this way Myc could contribute to overcoming Rb induced cell cycle arrest [86].

The aforementioned characteristics are hallmarks of the unique PSC cell cycle profile that facilitates long-term proliferation and possibly imposes differentiation blockades. Although not yet formally proven, these cell cycle properties are likely to be associated with mechanisms underpinning pluripotency.

With the collapse of c-myc levels on PSC differentiation, there is a reduction in expression of regulatory cyclins and upregulation of the cell cycle inhibitors $p21^{Cip1}$and $p27^{Kip1}$. This results in decreased CDK activity, effectively activating Rb and establishing cell cycle regulated activities [80, 81]. Significant restructuring of the cell cycle occurs so that the proportion of cells in G1 increases to >40% and the proportion of S-phase cells is reduced. The end result is a profile and generation time that is similar to that observed in many somatic cell types [80]. Therefore, Myc function is likely to be at the center of this regulatory network that maintains the characteristic PSC cell cycle.

Regulation of miRNAs

miRNAs add another layer of complexity to the regulation of pluripotent cells by fine-tuning gene expression [56, 87, 88]. Myc-regulated miRNAs in PSCs include the miR-17-92 cluster, members of the 290 family, and miRs-141, 200, 302, 338, 429 [46, 89, 90]. Through regulation of these miRNAs, Myc's reach may expand to a multitude of other targets to affect the PSC state. In support of this, several Myc-regulated miRNAs have established roles in self-renewal [89]. Indeed, deletion of c- and N-myc in PSCs results in downregulation of members of the miR-17-92 cluster. Upon Myc deletion and downregulation of the miRNA cluster, remodelling of the cell cycle occurs concomitant with differentiation [46]. This cluster may further extend Myc's control over PSC proliferation

through inhibiting cell cycle controllers such as p21, Rb2 and cyclin D1, which are regulated targets of the cluster [46, 91, 92].

The other core PSC regulators, Oct4, Sox2, and Nanog also regulate miRNAs, thereby refining gene expression in PSCs [56]. In fact, two groups of miRNA promoters with opposing function are directly bound by Oct4, Sox2, or Nanog in ESCs. The first group includes miRNAs found by Chen and colleagues who identified 14 unique miRNAs including members of the miR-290 and miR-302 families by comparing expression levels of miRNAs in mESCs, embryoid bodies and somatic cells [93]. Notably, the miR-290 family is one in which the Oct/Nanog/Sox2 and Myc circuits intersect. Judson et al. highlighted the significance of this cluster in establishment of pluripotency by showing that in the absence of exogenous Myc, expression of members of the cluster increases the efficiency of reprogramming to iPSCs by Oct4, Sox2 and Klf4 [94]. The second group of miRNA genes bound by Oct4, Sox2 and Nanog are also bound by polycomb-group proteins. These miRNAs are thought to be repressed targets and are induced upon differentiation [56]. One such repressed miRNA is Let-7. The Oct4/Sox2/Nanog and Myc circuits may converge on this miRNA to impede its expression in PSCs [56, 95]. Upon differentiation, Let-7 may also contribute to the decline in Myc levels since Myc is a *bona fide* Let-7 target [95].

Numerous additional miRNAs are also repressed by Myc. These include miRs-15a, 26a, 34a and 150 [96]. Many of these miRNAs are tumor suppressors through which Myc repression contributes to tumorigenesis [96]. Several Myc-repressed miRNAs are involved in promoting differentiation, while others oppose Myc directed PSC maintenance functions. For instance, miR-26a is involved in muscle differentiation, while members of the miR-15a cluster may block S-phase entry since a number of cell cycle regulators such as CDK6 are direct targets [97]. Thus Myc repression of these miRNAs may further contribute to pluripotency establishment and maintenance.

Myc's control of the PSC Epigenetic State

Chromatin organization and epigenetic regulation are generally thought to be important determinants of developmental potency. One striking feature about PSCs is their open chromatin structure with large regions of transcriptionally-active euchromatin [98–100]. It is often argued that the euchromatic state allows for many types of genes to be expressed, consistent with the general developmental plasticity of PSCs. Although it is difficult to demonstrate this directly, many pieces of evidence indicate that factors important for pluripotency are also involved in the epigenetic status of PSCs. Nanog and Oct4 for example, interact with epigenetic regulators such as the histone demethylase LSD1 and the Swi/Snf family member, Smarca4 [60]. Such interactions directly regulate histone methylation status and may mediate target gene accessibility by recruiting the esBAF chromatin-remodeling complex [101].

c-myc could also modulate chromatin state in PSCs, since several histone-modifying and Swi/Snf chromatin remodeling factors are c-myc transcriptional targets [58, 74]. Myc is also known to recruit a variety of epigenetic factors to target genes including DNA methyltransferases, histone-modifying enzymes and components of the chromatin remodeling machinery [102–104]. As Myc interacting partners include chromatin remodeling components and histone acetylases and deacetylases [79, 105–107], it is likely to globally influence chromatin state. Myc is also able to recruit various other HAT containing complexes including CREB binding protein, p300 and TRRAP, components of histone acetyltransferase complexes GCN5 and TIP60 [108, 109]. Recruitment of TRRAP complexes also implicates Myc in chromatin remodeling by members of the ATP dependent helicase complexes TIP48/TIP49 [105, 110].

In addition to acetylated lysine residues on histone H3 and H4, other histone marks such as trimethylation of lysine 4 of histone H3 (H3K4me3) are also associated with transcriptional activity. This modification is also abundant in PSCs [98, 99, 111]. Aside from genes containing these active marks, regulatory regions of certain developmental factors including Sox and Pax family members exist in a poised state. They contain bivalent domains containing both active H3K4me3 and repressive H3K27me3. These genomic regions are thus primed for activation upon receipt of the appropriate specification stimulus [112, 113]. A significant proportion of these genes are also marked by H3K27me3 establishing polycomb proteins as well as the core regulatory factors in this regulation [55, 61, 112].

E-boxes located around regions of H3K4 trimethylation are thought to serve as high affinity c-myc binding sites [114]. In ESCs, c-myc's binding sites significantly overlap with H3K4me3 [58], supporting Myc's relationship with the unique PSC epigenetic state. Intriguingly, Myc also interacts with, and inhibits H3K4 demethylases of the Lid/Jarid1 family [37]. This is one possible mechanism by which Myc may be involved in fine-tuning of the expression of genes involved in maintaining self-renewal.

Myc binding is also inversely associated with the heterochromatin associated H3K27me3 mark [58]. This goes along with the finding of few overlapping sites within genome wide maps of Myc and polycomb repressive complex 2 that mediates H3K27 methylation [74]. Although Myc binding correlates with the presence of certain epigenetic marks [102, 114], it remains unclear whether Myc establishes these marks or is recruited as a consequence of them.

Histone modifications associated with pluripotency change dramatically during differentiation and coincide with decreased Myc activity. Activating marks such as H3K4me3 decrease and those associated with repression, such as H3K9me3 and H3K27me3, become more prevalent. Similarly, repressive marks are concurrently removed from genes that are required for lineage specification. Altogether, this results in more compacted heterochromatin and a more repressive gene expression signature. Promoters of the important pluripotency factors Oct4 and Nanog also undergo DNA methylation to impede active transcription [62, 98, 99, 115]. It will be important to establish if Myc is involved specifically in regulation of DNA demethylation of pluripotency genes, via AID-dependent or independent mechanisms.

Myc: At the Center of Reprogramming

The regulatory functions assigned to Myc so far, firmly establish it as being critical for maintenance of pluripotency. Myc is also one of the original four factors used in the reprogramming of somatic cells to the pluripotent state [4] (Figure 2). Significant effort has gone into delineating the function of Myc in the dedifferentiation process. Myc has well-established roles as an inhibitor of differentiation [116], and blockage of endoderm transcriptional programs [46] may be one mechanism by which Myc supports the dedifferentiation process. Another role for Myc in pluripotency establishment could be to facilitate access of the other reprogramming factors to target loci. This would involve recruiting chromatin modifiers such as histone acetyltransferases or histone/DNA demethylases [4, 75]. Remodeling of gene expression patterns during early reprogramming is likely to be dependent on Myc's ability to impose global epigenetic change. Recent studies support this model and indicate that c-myc is required to extinguish fibroblast-specific genes during the early stages of reprogramming [117]. This is supported by experiments showing that the histone deacetylase inhibitor, valproic acid, can substitute for Myc in iPSC generation [118]. Other compounds that modulate the DNA or chromatin status, such as BIX01294 and azacytidine also affect reprogramming efficiency [119, 120].

These observations again highlight the importance of epigenetic regulation in the establishment and maintenance of the pluripotent state.

The catalytic subunit of telomerase, TERT, is a transcriptional target of Myc and so it is likely that PSCs maintain telomeric length by high TERT activity [5, 121]. This is similar to the situation in immortalized cancer cells where increased Myc activity is associated with a coinciding increase in telomerase activity [122, 122, 123]. Elevated TERT expression is another key event associated with the establishment of iPSCs [5, 121]. Regulation of telomerase activity is likely to be another Myc-dependent event during reprogramming.

Another likely role for Myc in reprogramming process involves cell cycle progression. A number of reports clearly indicate a direct link between a cell's proliferative capacity and its capacity for reprogramming (Table 1) [124, 125]. Myc has long been associated with regulation of cellular division. In somatic cells, exogenous c-myc expression promotes entry from quiescence into the cell cycle. Myc overexpression often results in transformation and in many cases, accelerated progression through the G1 phase [126–128]. In extreme cases, such as in breast cancer cells with elevated cyclin E expression, this leads to a cell cycle profile more similar to that of PSCs. Additional reports link Myc's role in cell cycle control to reprogramming. For example, inhibition of the Cdk inhibitor $p21^{Cip1}$ and its upstream regulator, p53, accelerates reprogramming [129, 130]. In this scenario, Myc could inhibit the Cip1 gene at the transcriptional level or post-transcriptionally through regulation of miRNAs [131–133]. Myc may therefore be the link between cell cycle control and establishment and maintenance of PSCs. Indeed, cell cycle remodeling occurs in a stepwise fashion during reprogramming and partially reprogrammed cells have a profile intermediate between somatic cells and iPSCs [134].

Yet another mechanism by which Myc impact reprogramming is through DNA replication. c-myc regulates DNA replication by increasing activity at replication forks [30]. During the expanded S-phase, progressive displacement of repressive factors and exposure to other reprogramming factors may facilitate the genome-wide remodeling of transcriptional programs.

Reprogramming has now been achieved without exogenous Myc. For example, reprogramming has been achieved using Lin28 and Nanog in place of Myc [135]. It is likely however, that Myc is still critical under these conditions since endogenous Myc is present in all cell types. The function of Nanog in maintaining pluripotency was previously discussed and interestingly enough, Nanog was recently implicated as a low affinity c-myc target in mESCs [136]. Further assessments are required to determine if this is a functionally relevant interaction. It should also be mentioned that Myc is a Lin28 target [58]. Lin28 is a RNA binding protein that possibly stabilizes the transcripts [137] of key reprogramming factors. If Lin28 acts to stabilize Myc transcripts, this could indirectly propagate Myc-Lin28 function through somewhat of a feed-forward mechanism. In sum, the data discussed here illustrate the profound role of Myc in the establishment and maintenance of PSCs.

Myc: A Therapeutic Impediment?

PSCs share many common properties with cancer cells including senescence escape and immortality. However, unlike stem cell populations which are under strict biological constraints, tumors result when such control has gone awry. Combinations of the following processes are common in cancers: ectopic growth, unlimited proliferation, differentiation inhibition, and escape from apoptotic control [18,138, 139]. Not surprisingly, Myc has been linked to promoting such processes in normal stem cells [46, 67, 68]. Therefore, it is easily envisioned that deregulation of Myc is tightly linked to aberrations in these functions in cellular transformation.

Tumors are phenotypically variable and this depends on the cell of origin [140]. The tumorigenic capacity of iPSCs (and ESCs) is exemplified by the formation of tumors upon injection into immunodeficient recipients. These teratomas, discombobulated masses of cells, contain cellular derivatives of the embryonic germ layers, and demonstrate the differentiation potential of PSCs [2, 141]. Other malignancies consist primarily of poorly differentiated cells. This is often the case with cancers of the blood, which is normally repopulated by the rapidly dividing transit amplifying cells [140, 142].

Interestingly, the recent advances in iPSC generation, where Myc promotes reversion to a more primitive undifferentiated state provides indirect evidence for Myc's capacity to generate tumors primarily consisting of undifferentiated cells. Reprogramming can be viewed as similar to a transformation process. However, tumors are not the primary result as other reprogramming factors balance Myc activities to lend control and pluripotency specification. Specifically, Klf4 balances Myc promoted proliferation though regulation of cell cycle inhibitors which are normally inhibited by Myc [143]. Oct4 and Sox2 (and Nanog) function to direct the activation of pluripotency genes and repression of lineage targets as discussed previously [57].

Transformation may occur in differentiated cell types to generate cells that have regained some self-renewal capacity. There is also evidence that some cancers originate from rogue populations of stem cells that have overridden biological checks [140]. Such cancer stem cells may arise from normal stem cells accumulating genetic errors leading to their transformation. A genetic insult to result in amplified Myc expression could be the driving force behind such transformation. Indeed, Myc is a contributing factor in maintenance of some cancer stem cell populations, as is the case with hepatocellular carcinoma and glioma cancer stem cells [144, 145].

Any slight perturbations in Myc expression can have disastrous results in biomedical applications, as evidenced by tumors that develop in 20% of mice generated from iPSCs [9]. Reactivation of the Myc transgene was observed in mice in which the tumors arose, and was the likely driving force of the resultant tumors. Similarly, the transplantation of any undifferentiated PSCs, or differentiated derivatives that display even slight elevation of Myc could potentially have disastrous consequences for recipient patients. However, as mentioned previously Myc is a necessary factor for maintenance of fundamental PSC characteristics, therefore complete inactivation of Myc will not be suitable even if the aim is to attenuate cancer risk. What is encouraging though is that Myc-dependent tumors regress with inactivation of Myc [146, 147]. This suggests that PSC cell therapy is feasible. This will likely require careful monitoring of Myc levels in cells intended for therapeutic use, and avoiding the use of exogenous Myc under any circumstance, as even small amounts are likely to skew the delicate balance.

Future Perspective

Myc is a well-established regulator of transcription but much still remains unanswered in relation to how it maintains PSCs. A full understanding of Myc's function will be significant for development of therapeutics derived from PSCs. Although studies of Myc in maintenance of self-renewal and pluripotency have provided some insight into their roles in peri-implantation development, further studies on each family member are required. Key questions that remain include: what is the phenotype of the triple knockout during the pre-/peri-implantation period? Are precise levels of c- or N-myc required during this period or will a certain threshold of total Myc suffice? What is the function, if any, of L-myc in the choice between self-renewal and differentiation? How is each family member regulated at the transcriptional, post-transcriptional and translational level? What proteins are present in

Myc complexes, and how are these interactions mediated? How does N-myc redundancy account for the placental recovery in the c-myc null background? Furthermore, instances where each member has a specific role in specification of embryonic germ layers and their immediate derivatives is feasible, and requires further investigation.

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Executive Summary

Myc: Critical for Embryonic development

- c-myc and N-myc are required for embryonic development prior to implantation
- L-myc cannot functionally substitute for c- and N-myc.

Regulation of PSCs: Complex Circuitry

- Control of the PSC state is complex and involves the coordination and functional interaction of key regulators.
- Myc collaborates with other key core PSC regulators to maintain the pluripotent state.

Myc: Pleiotropic regulator of the PSC state

- Throughout the genome, Myc binds genic and intergenic regions to regulate the expression of thousands of genes and ncRNAs.
- It is a key regulator of the PSC cell cycle.
- Recent evidence has defined its role as a regulator of the epigenome.
- Due to its control of DNA replication, it facilitates resetting of the epigenome to establish and sustain pluripotency.
- It maintains telomere lengths through promoting expression of telomerase.

Myc: A Therapeutic Impediment

- Careful monitoring of Myc levels is essential for any PSC derived therapeutic.
- Exogenous activation of Myc expression is ill-advised for stem cell therapies.

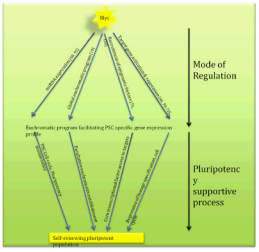

Figure 1. Roles for Myc in maintaining PSCs

Myc regulates establishment and maintenance of pluripotency using diverse mechanisms.

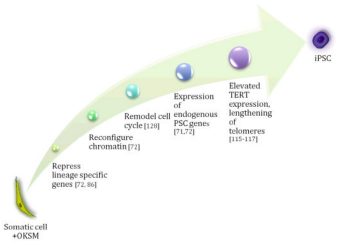

Figure 2. Myc participates in many aspects of the multistep process to reprogram somatic cells to the pluripotent state

Table 1

iPSC generation in the presence and absence of Myc.

Reprogramming factors	Cell type	Reference
iPSC generation with Myc		
OKSM	Mouse fibroblasts	[4, 9, 148]
	Mouse gastric epithelial cells	[149]
	Mouse hepatocytes	[149]
	Mouse B-lymphocytes	[150]
	Mouse neural stem/progenitor cells	[151, 152]
	Mouse pancreatic beta-cells	[153]
	Human fibroblasts	[5][154]
	Human bone marrow mesenchymal cells	[155]
	Human keratinocytes	[156]
	Human adipose stem cells	[157]
	Human cord blood stem cells	[158]
	Human blood progenitor cells	[159]
	Marmoset fibroblasts	[161][162]
	Rat primary ear fibroblasts	[162]
	Rat bone marrow cells	[162]
	Pig fetal fibroblasts	[163]
	Dog embryonic fibroblasts	[164]
	Rabbit stomach and liver cells	[165]
OKM	Mouse neural stem cells	[151, 152]
OMS	Mouse neural stem cells	[152]
OM	Mouse neural stem cells	[152]
iPSC generation without Myc		
OSKLN	Human aortic vascular smooth muscle cells	[166]
OSLN	Human fibroblasts	[167]
	Human cord blood endothelial cells	[168]
OKS	Mouse embryonic fibroblasts	[169, 170]
	Mouse hepatocytes[118]	[149]
	Mouse neural stem cells	[152]
	Human keratinocytes	[156]
	Human adipose stem cells	[171]
	Human cord blood stem cells	[158]
OKS (valproic acid)	Human fibroblasts	[172]
OKN	Human fibroblasts	[167]
OKL	Human fibroblasts	[167]
OK	Mouse neural stem cells	[152]
OS (valproic acid)	Human dermal fibroblasts	[118]
	Human cord blood stem cells	[158]
O	Mouse neural stem cells	[173]

O:Oct4, S:Sox2, K:Klf4, M:Myc, N:Nanog